RESEARCH PAPER

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Impact of *ERBB2* mutations on in vitro sensitivity of bladder cancer to lapatinib

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Lapatinib, a dual tyrosine kinase inhibitor of ErbB1 and ErbB2, shows a clinical benefit in a subset of patients with advanced urothelial bladder cancer (UBC). We hypothesized that the corresponding gene, *ERBB2*, is affected by mutations in a subset of UBC and that these mutations impact ErbB2 function, signaling, UBC proliferation, gene expression, and predict response to lapatinib. We found *ERBB2* mutations in 5 of 33 UBC cell lines (15%), all of which were derived from invasive or high grade tumors. Phosphorylation and activation of ErbB2 and its downstream pathways were markedly enhanced in mutated cell lines compared with the *ERBB2* wild-type. In addition, the gene expression profile was distinct, specifically for genes encoding for proteins of the extracellular matrix. RT112 cells infected with *ERBB2* mutants showed a particular growth pattern ("mini-foci"). Upon treatment with lapatinib, 93% of these "mini-foci" were reversed. The sensitivity to lapatinib was greatest among cell lines with *ERBB2* mutations. In conclusion, *ERBB2* mutations occur in a subset of UBC and impact proliferation, signaling, gene expression and predict a greater response to lapatinib. If confirmed in the clinical setting, this may lead the way toward personalized treatment of a subset of UBC.

Introduction

Urothelial bladder cancer (UBC) is the second most common genitourinary malignancy in men and the ninth most common in women, with more than 72000 new cases and 15000 deaths expected every year in the US.[1-3] About 75% of patients present with non-muscle-invasive UBC, which is treated by transurethral resection and intravesical therapy.[4] In all, 50% to 80% of these tumors will recur, and up to 30% will progress to a muscle-invasive stage,[5] ultimately requiring radical cystectomy. In addition, 20% to 30% of newly diagnosed patients have muscle-invasive UBC, which is primarily treated with radical cystectomy.[2,6] Following curative surgery, approximately 50% of these patients progress to metastatic disease.[6] At present, there are no widely accepted adjuvant chemotherapeutic regimens that significantly delay recurrence and improve survival.

Moreover, chemotherapy in the metastatic setting is seldom if ever curative. Therefore, there is a need for safe and effective systemic therapies for UBC. In the era of personalized medicine, biomarker driven targeted therapies may allow delivery of effective therapy to those patients most likely to benefit from it, sparing their side effects to those unlikely to respond. In the last years, many efforts have been made to develop and test agents that impact the activity of epidermal growth factor receptor (EGFR) family members.[7-9] Among these, ErbB1 (the epidermal growth factor receptor) and ErbB2 (HER2/Neu) belong to the Erb family of growth factor receptor tyrosine kinases (TKI), which mediate proliferation, migration and differentiation[10,11] through two principal pathways: Ras-Raf-MAPK and PI3K/Akt/mTOR. ErbB1 and ErbB2 are highly expressed in some UBC[12-14] and may, therefore, represent an attractive therapeutic target.[15] Both have been shown to be associated with prognosis and metastasis of UBC.[11,13]

Lapatinib is a dual TKI of ErbB1 and ErbB2.[16-19] In vitro studies on UBC cell lines showed that lapatinib has cytostatic and cytotoxic effects and acts synergistically with conventional chemotherapies.[16,20] An uncontrolled open-label phase 2 trial evaluated lapatinib as second line therapy in locally advanced or metastatic UBC. Although the study was considered to be negative, a clinical benefit rate of >30% was seen. Importantly, clinical benefit correlated with ErbB2 overexpression within the tumor and lapatinib was well tolerated.[21]

We hypothesized that the corresponding gene of ErbB2, *ERBB2*, is affected by mutations in a subset of UBC and impacts ErbB2 function and subsequently UBC progression and response to lapatinib. This could improve patient selection for lapatinib and lead the way toward personalized treatment of UBC. Comparable work has previously been done in breast cancer, where

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Table 1. Six *ERBB2* mutations were detected in 5 of 33 UBC cell lines. The protein domain is indicated

Position	Cell line	Mutation	Domain
37856533	VM-CUBIII	(CTC)L15F(TTC)	Signal
37864775	DSH1	(CGA)R143Q(CAA)	Extracellular I
37866661	DSH1	(GAC)D277H(CAC)	Extracellular II
37868207	DSH1	(TCC)S310F(TTC)	Extracellular II
	5637	(TCC)S310F(TTC)	Extracellular II
37879582	VM-CUBI	(TCC)S653C(TGC)	Transmembrane
37879657	J82	(CGG)R678Q(CAG)	Transmembrane

lapatinib received approval by the FDA for ErbB2-positive disease.[22] To test this hypothesis, we screened 33 UBC cell lines for *ERBB2* mutations by RNA sequencing. We then studied the impact of *ERBB2* mutations on ErbB2 signaling, in vitro growth, gene expression and response to lapatinib.

Results

Screening of bladder cancer cell lines for ERBB2 mutations

Screening by RNA sequencing followed by bioinformatic analysis showed *ERBB2* mutations in 5 of 33 UBC cell lines (15%). Three distinct mutations were found in DSH1, while one mutation was found in VM-CUBI, VM-CUBIII, 5637, and J82 (**Table 1**). All cell lines with *ERBB2* mutations are derived from invasive or high grade tumors. Only T24 showed a mutation in *H-RAS* ([GGC]G12V[GTC]).

Impact of ErbB2 on proliferation

The impact of *ERBB2* mutations on proliferation was evaluated in the 5 UBC cell lines (VM-CUBI, J82, 5637, DSH1, and VM-CUBIII) with mutations and compared with 4 UBC cell lines with wild-type *ERBB2* (UM-UC14, T24, BC3C, and SW780). Three *ERBB2*-targeting shRNAs (sh1, sh4, and sh6) were used to knockout *ERBB2* in the cell lines. Sh4 and sh6 showed significant effects in 3 of the cell lines with mutations (VM-CUBI, 5637, and DSH1) and in 2 belonging to the wild-type group (BC3C and SW780). Sh1 decreased the ErbB2 more effectively and inhibited cell proliferation in all cell lines (**Fig. 1A–C**).

Activation status and cell growth pattern by ERBB2 mutational status

We then tested whether protein phosphorylation differed in 293FT and RT112 cell lines with *ERBB2* mutations and *ERBB2* wild-type. We first evaluated the activation of 293FT cells by monitoring the phosphorylation on the tyrosine residues 1221/1222 and 1248. Our experiments showed enhanced phosphorylation of cells with the mutations S310F, S653C, R678Q, or D277+S310F than for L15F, R143Q, or D277H, although phosphorylation of the tyrosine residues was still visible (data not shown).

RT112 cells were infected with lentiviral vectors containing the *ERBB2* wild-type or mutated sequences. Phosphorylation and thus activation of ErbB2 tyrosine residue 1248 as target of ErbB2 tyrosine kinase (ErbB2-Y1248), ErbB3-Y1197, EGFR-Y1068, Shc-Y239/40, and p-Erk1/2 were assessed. The western blot conducted on RT112 cells showed that, compared with wild-type *ERBB2*, phosphorylation of ErbB2 and downstream targets were increased in some mutant *ERBB2* (S310F, S653C, R678Q, and D277H$^+$S310F, **Fig. 2A**). L15F, R143Q, and D277H showed minor effects, but the latter increased phosphorylation together with S310F.

There were also marked changes in the growth pattern of RT112 between the distinct *ERBB2* mutations. RT112 cells infected with *ERBB2* mutants S310F, S653C, and D277H$^+$S310F grew in groups of cells that were strongly aggregated to each other, a process called "mini-foci" formation. In confocal microscopy, the borders of cells in the "mini-foci" were visible in S310F and S653C, but blurred in D277H$^+$S310F (**Fig. 2B**).

Finally, the infected RT112 cells were cultured for 7 d and subsequently incubated with AZD6244 (selumetinib), lapatinib, or afatinib. After incubation, lapatinib reversed 93% and afatinib 96% (P = n.s.) of the "mini-foci" formation in cells expressing the three *ERBB2* mutants compared with a rate of reversal < 40% with AZD6244 ($P < 0.01$, **Fig. 3A and B**). Both lapatinib and afatinib had similar growth-inhibiting effects in RT112 and reduced pAKT(S473) to a similar extent.

Gene expression by ERBB2 mutational status

We evaluated gene expression in RT112 cells expressing the vector, wild-type or mutant *ERBB2* (S310F and S653C). RNA sequencing data were analyzed by hierarchical clustering. We found that S310F and S653C were assigned to one cluster, while the wild-type and the vector were assigned to the other cluster (**Fig. 4A**).

The differentially expressed genes ($P < 0.05$, fold changes > 2 or < 0.5) among the arrays were assigned into clusters according to their function (**Fig. 4B–G**). The most significant changes were observed in extracellular proteins (group I). According to this analysis, *ERBB2* S310F and S653C affected the extracellular matrix, cell adhesion, motility, environmental signals from growth factors, cytokines, hormones, and epithelial differentiation (groups II–VI). These six clusters contained 209 differentially expressed genes for *ERBB2*-S310F and 134 for *ERBB2*-S653C. Expression levels of selected extracellular genes were assessed by qPCR, and results were consistent with RNA sequencing (R = 0.99).

Response to lapatinib by ERBB2 mutational status

Cluster analysis according to the logarithm of IC_{50} for lapatinib using city-block similarity metrics and complete linkages. According to the IC_{50} after three days of treatment, three groups of sensitivity were distinguished (**Fig. 5**). Cells of group III exhibited the highest sensitivity to lapatinib. IC_{50} were 25–100 times lower than those of group I (**Fig. 5A**). RT112 with effective *ERBB2* mutation (VM-CUBI, J82, 5637, and DSH1) were among the most sensitive cell lines. The association between the *ERBB2* mutation status and the sensitivity to lapatinib was statistically significant (P = 0.0279, **Fig. 5B and C**). RNA expression levels of EGFR and *ERBB2* did not correlate with the sensitivity to lapatinib (R = –0.23 and –0.24, respectively).

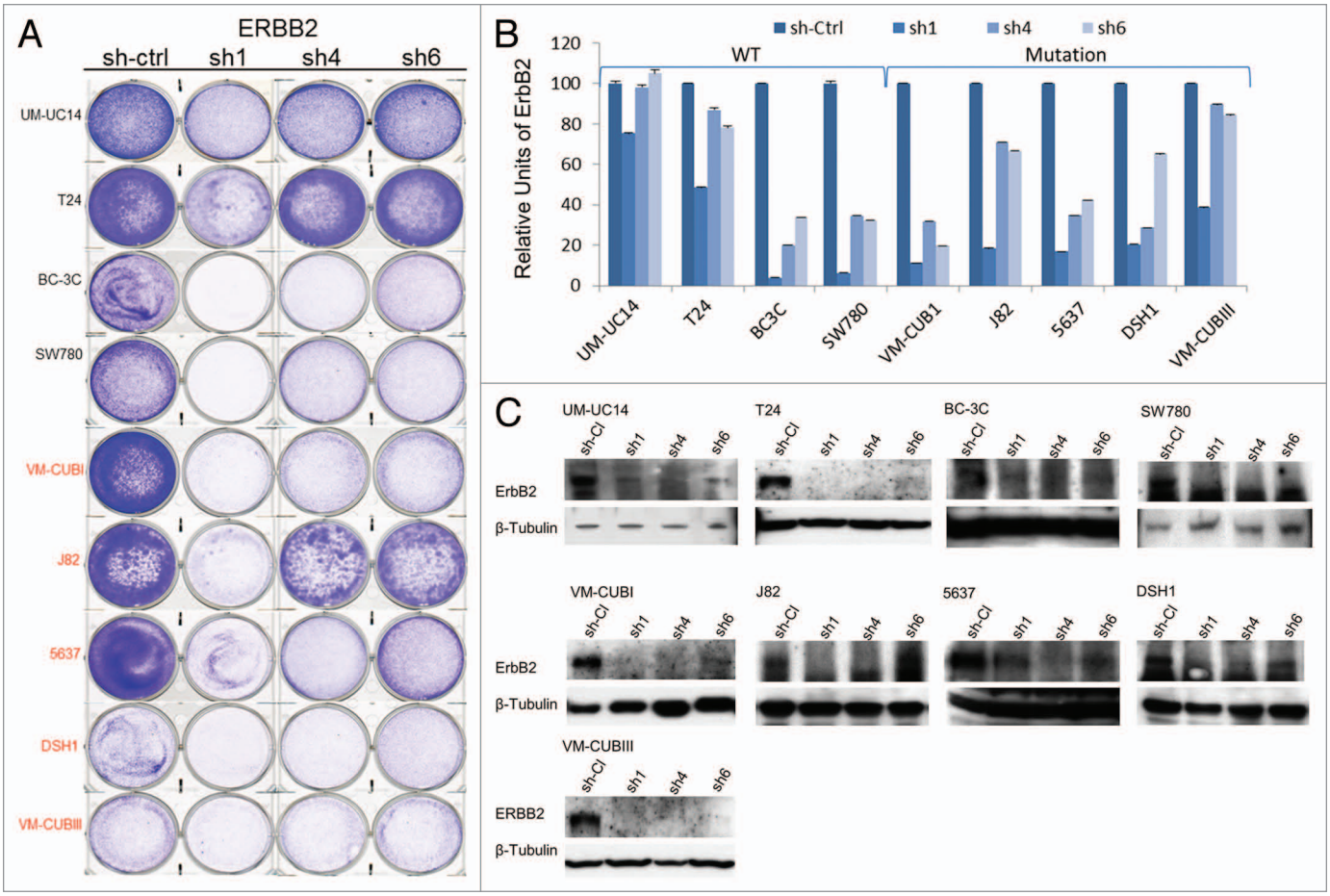

Figure 1. Knockdown of *ERBB2* in five cell lines with and in four cell lines without *ERBB2* mutations. Five days after infection with shRNA, cells were fixed and stained by crystal violet (**A**). (**B**) Normalized (sh-Ctrl) relative quantification of cells. (**C**) Western blot for ErbB2 and β-tubulin. Data show the essential role of ErbB2 in some cell lines.

Discussion

We screened UBC cell lines for *ERBB2* and *H-RAS* mutations and evaluated their impact on proliferation, signaling, growth, gene expression, and response to lapatinib. We found *ERBB2* and *H-RAS* mutations in 15% and 3% of cell lines, respectively, all of which derived from invasive or high grade tumors. Phosphorylation and thus activation of ErbB2 and its downstream pathways was markedly enhanced in mutated cell lines compared with *ERBB2* wild-type. In addition, the gene expression profile was distinct in mutated cell lines, specifically for genes encoding for proteins of the extracellular matrix. RT112 cells with infected *ERBB2* mutants showed a particular growth pattern ("mini-foci"). Incubation with lapatinib reversed 93% of these "mini-foci" and the sensitivity to lapatinib was greatest among cell lines with *ERBB2* mutation.

Activating ERBB2 mutations

The genetic basis of UBC is well characterized.[23] Dysregulation of ErbB2 by gene amplification or somatic mutations has been linked with the development and progression of various malignancies, including UBC.[24,25] According to data provided by the "Catalogue Of Somatic Mutations In Cancer", the prevalence of somatic *ERBB2* mutations in UBC is around 5%, which is the highest among all tumor entities.[26] In the current study on UBC cell lines, the prevalence was about 3-fold higher. This is likely due to selection bias, as cell lines are commonly derived from high grade or metastatic tumors and therefore do not reflect the true stage and grade distribution in the clinical setting.

We found three mutations of *ERBB2* in the extracellular domain II (ECD2), which acts as a bridge between the two ligand-interacting domains ECD1 and ECD3, and as a dimerization arm in the receptor-receptor interaction.[25] Previous studies showed 35 mutations in ECD2, among which G309A/E, S310F/Y, C311R, E321G, and C334S were identified as driver mutations.[10,27] Among these, S310F was most commonly found in malignant tumors, including lung, colon, ovary, stomach, breast and UBC. In line with these data, we identified S310F in two cell lines. Greulich et al.[27] showed that the change in the amino acid from serine to voluminous phenylalanine induces hyperactivation. Similarly, we show that the *ERBB2* mutant induced the activation of the ErbB2 signaling transduction and remarkable changes in gene expression, which subsequently led to changes in proliferation, growth pattern and response to lapatinib.

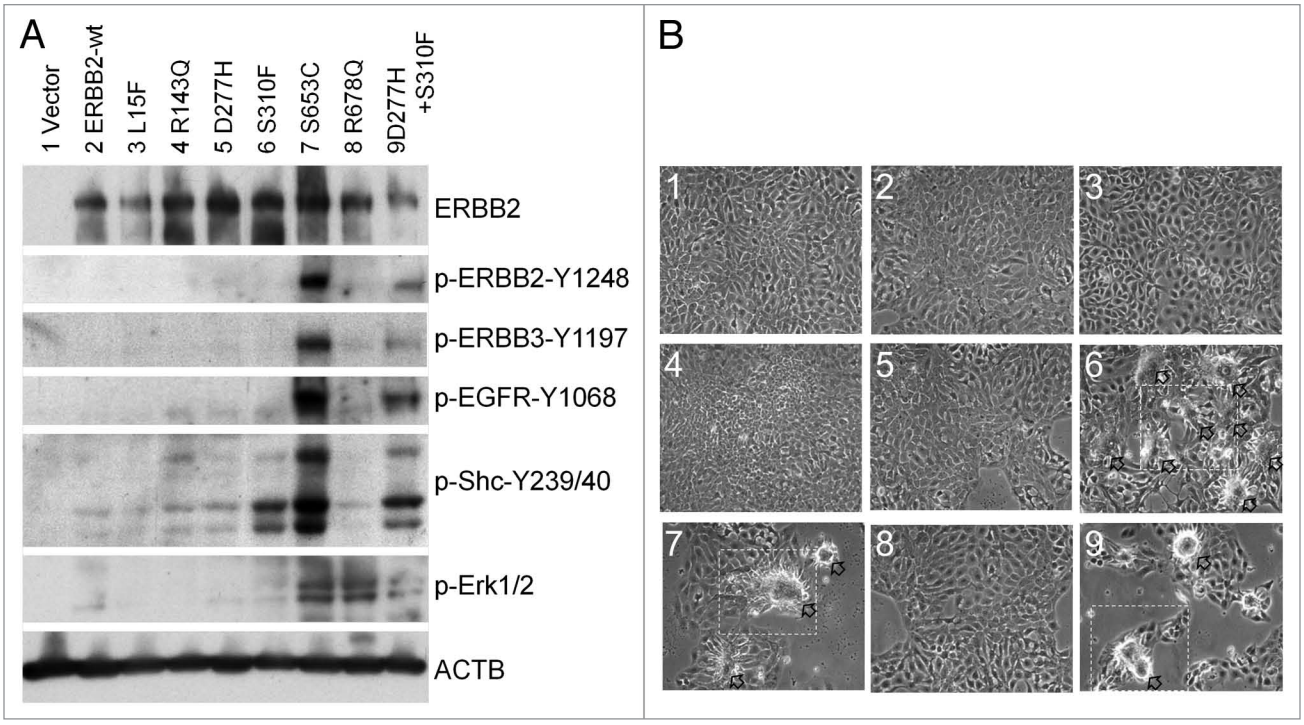

Figure 2. (**A**) Activation/phosphorylation of ErbB2 according to mutational status. Western blot for the protein phosphorylation in RT112 cells infected with a vector, wild-type or mutant *ERBB2*. (**B**) Activation/phosphorylation of ErbB2 according to mutational status. Formation of "mini-foci" of infected RT112 cells: 1, vector; 2, *ERBB2*-wild type; 3, *ERBB2*-L15F; 4, R143Q; 5, D277H; 6, S310F; 7, S653C; 8, R678Q; 9, D277+S310F. Arrows point to "mini-foci".

The mutation D277H has not been described in tumor samples before. Our data suggest that this mutation does not affect the activity of ErbB2 alone; but enhances its activation together with S310F. We demonstrate these effects at the level of ErbB phosphorylation and on stably infected cells.

Two mutations were found in the transmembrane domain. Studies show that the transmembrane domain actively regulates receptors by structural adaption for dimerization, selection of dimerization partners and dimer stabilization.[28-30] Bargmann and Weinberg[31] identified the V664E mutation in the rat, which was discovered in naturally induced tumors, however, this mutation has never been observed in human tumor samples. So far, three mutations were found in human tumor samples,[26] none of which has been further characterized. We found S653C in the cell line VM-CUBI, which led to activation of signal transduction and downstream gene regulation. We hypothesize that this mutation acts through the formation of disulfide bonds between the mutant cysteines, comparably with the mutations in the transmembrane domain of FGFR3.[32,33] To date, there is no further report of this mutation in patient samples. The mutation R678Q is located near the transmembrane domain. Four cases with this mutation are documented,[26] but we and others were not able to show any gain-of-function consequence.[10]

The tyrosine kinase domain contains 65% of all activating *ERBB2* mutations in human tumor samples,[26] the majority of which are insertions.[10,27,34] Interestingly, we did not find any mutation in this area, indicating a distinct distribution of *ERBB2* mutations in UBC.

Gene expression by activating ERBB2

We found that ErbB2 is essential to some UBC cell lines and that activating *ERBB2* mutations alter expression of a wide range of genes. The largest group of these genes comprised those encoding for extracellular proteins. The other clusters were related to cell differentiation and change the growth pattern of cells, which may explain the formation of "mini foci". The observed gene expression pattern differs substantially from the one in breast cancer, where mutant or overexpressed *ERBB2* induces epithelial-to-mesenchymal transition.[35]

Response to lapatinib and personalized medicine

In the past 30 y, no significant improvements have been made in systemic therapy of advanced UBC. As there are many biological pathways impacted, it is unlikely that a single drug will be effective in the majority of patients with advanced UBC. Systemic therapy should therefore be tailored for each patient, and the analysis of gene mutations, gene expression or protein expression within the primary tumor and metastases represents an option to achieve this personalized treatment.

We found *ERBB2* mutations in 15% of cell lines, which is close to the clinical benefit rate reported by Wülfing et al.[21] We found six different mutations in five cell lines, three of which were observed in DSH1. RT112 cells containing effective mutations (VM-CUBI, J82, 5637 and DSH1) showed response to lapatinib in vitro, and the greatest response was observed in the three mutations from DSH1. Taken together, our study generates the hypothesis that UBC harboring *ERBB2* mutations may be more susceptible to lapatinib and may thus more likely respond

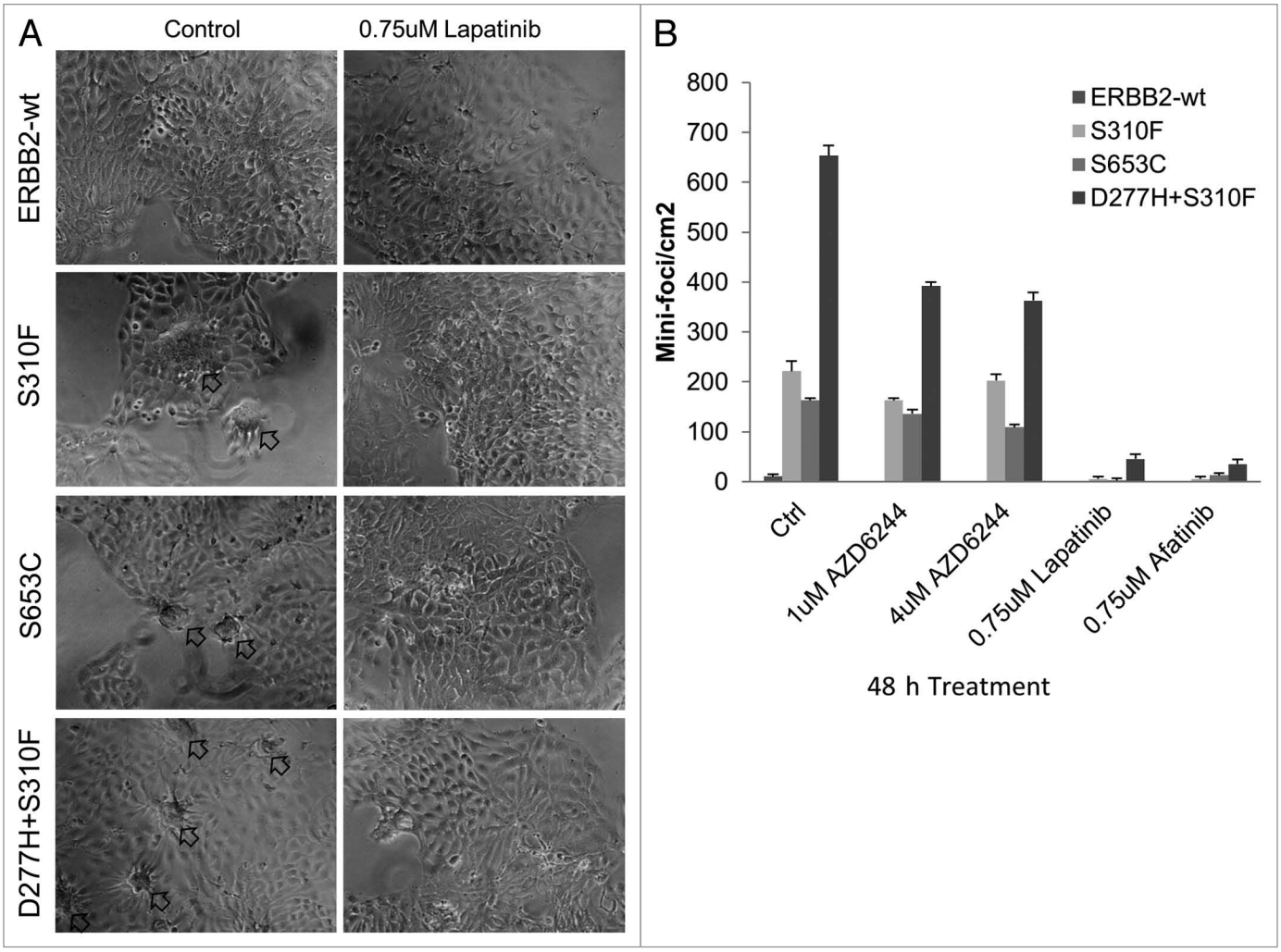

Figure 3. (**A**) Effects of 0.75uM lapatinib on "mini-foci" formation (arrows). (**B**) Effects of AZD6244, lapatinib and afatinib on the density of "mini-foci".

to this drug. In vivo studies or retrospective tissue based studies from patients, who received lapatinib, are necessary.

Overexpression of ErbB2 has been described in many solid cancers. Gunes et al.[36] showed that the expression of ErbB2 was 4.72-fold increased in UBC compared with normal bladder tissues. Schneider et al.[37] demonstrated that in micro-papillary urothelial carcinoma, amplification of *ERBB2* was associated with a 3-fold increased risk of death. Subsequently, lapatinib has been tested together with other drugs in vitro. McHugh et al.[20] investigated lapatinib with gemcitabine and cisplatin chemotherapy (GC). The authors used the UBC cell lines J82 and RT112, which are characterized by low and high expression of ErbB2, respectively. Lapatinib potentiated the efficacy of GC in both J82 and RT112, indicating a potential combination of these drug regimens. In a further study, the authors analyzed the effects of lapatinib with gemcitabine, paclitaxel and cisplatin.[16] They showed that in RT112 cells, there was accumulation of sub-G_1 cell population due to phosphorylation of the p53-Ser46. This finding indicates that the effects seen by addition of lapatinib may depend from the genotype. One may speculate that a variation changing Ser46 in another amino acid would not lead to activation and accumulation of the sub-G_1 cell population.

In several other cancer entities, personalized systemic treatment is already reality. For example, Lynch et al.[38] explored gefitinib in non-small cell lung cancer (NSCLC), which targets EGFR. The authors analyzed the EGFR gene sequence and found that eight of nine patients that responded to gefitinib had mutations in the catalytic domain while none of seven non-responding patients had mutations. KRAS gene mutations were shown to be predictive and prognostic for colorectal cancer and NSCLC.[39,40] Together with BRAF mutations, they are analyzed as part of clinical routine.[41] Mutations in BRCA1, BRCA2, and CDH1 are routinely assessed for breast cancer screening, and trastuzumab is given to treat tumors that overexpress ErbB2.[42]

Afatinib is an irreversible ErbB family blocker, which was recently approved by the FDA for the treatment of NSCLC patients with *ERBB2* deletions in exon 19 and the R858L mutation in exon 21. In UBC, afatinib was shown to suppress the activity of ErbB2 in vitro and in vivo in presence and absence of mutations, and to act synergistically with radiation.[7] Guo et al.[43] showed no difference in cell growth inhibition of UBC

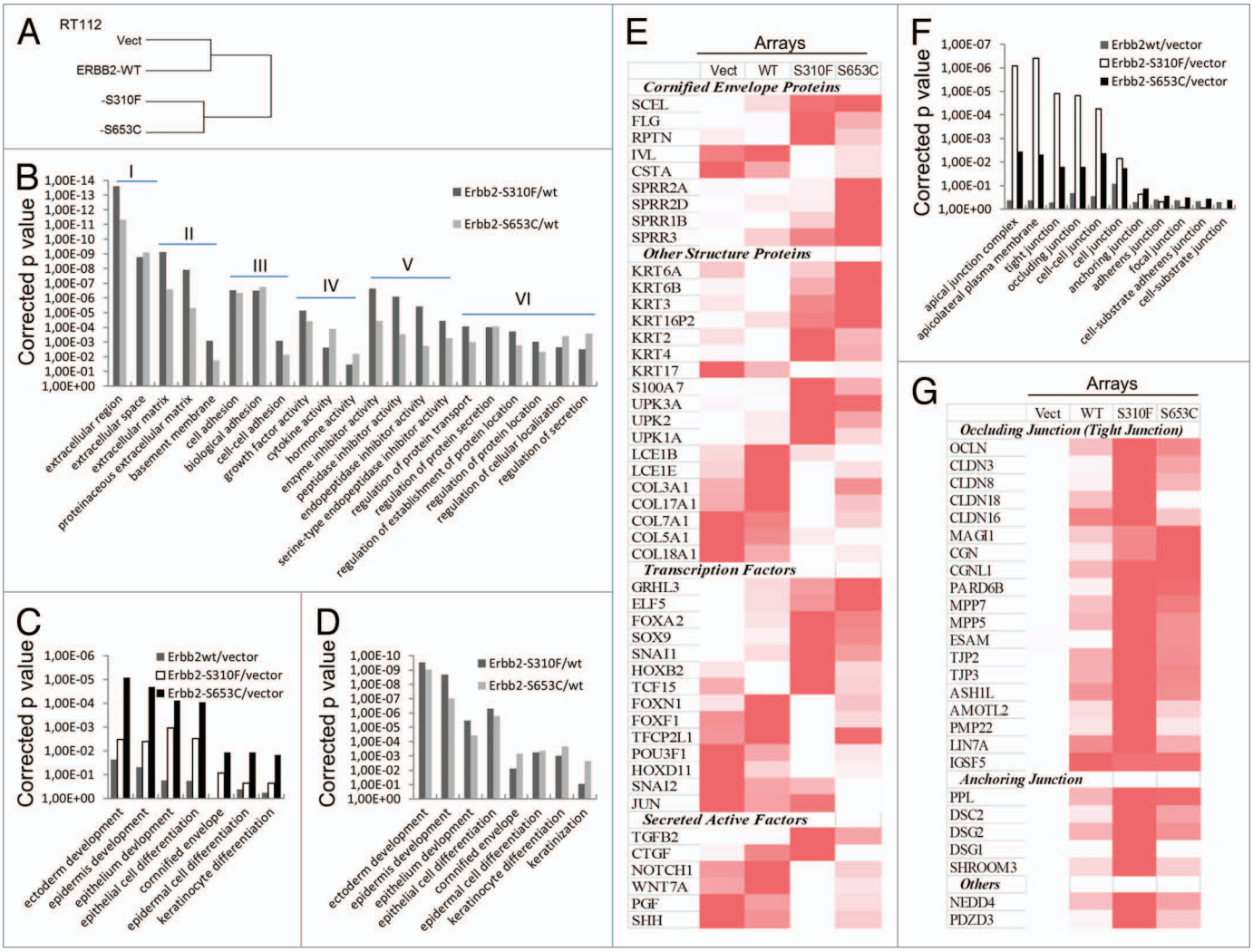

Figure 4. Gene expression by *ERBB2* mutational status. (**A**) Array tree for the gene hierarchical clustering of the gene altered in RT112 cells with either vector, wild type (WT), or mutant *ERBB2* (S310F, S653C). (**B, C, D, and F**) Association of the clusters with the gene expression. The most significant changes were observed in extracellular proteins, with the Gene Oncology (GO) terms "extracellular region" or "extracellular space" (group I). They were further clustered into extracellular matrix (group II; GO terms "extracellular matrix", "proteinaceous extracellular matrix", and "basement membrane"), cell adhesion (group III; GO terms "cell adhesion", biological adhesion", and "cell–cell adhesion"), secreted active molecules (group IV; GO terms "growth factor activity", "cytokine activity", and "hormone activity") and enzyme inhibitors (group V; GO terms "enzyme inhibitor activity", "peptidase inhibitor activity", "endopeptidase inhibitor activity", and "serine-type endopeptidase inhibitor activity"). The transport, secretion and localization of extracellular proteins is supported by regulators of protein secretion (group VI; GO terms "regulation of protein transport", "regulation of protein secretion", "regulation of establishment of protein localization", "regulation of protein localization", "regulation of cellular localization", and "regulation of secretion." Another two gene clusters among the most altered ones were related to epithelial differentiation (**C**) When the differentially expressed genes between mutants and wild-type were analyzed, the cluster was more significantly altered (**D**). (**E and G**) Heat map of gene expression levels from RNA sequencing. TF, transcription factor; SF, signaling factor.

and reduction of pAKT(S473), which is in line with our data. Although irreversible inhibitors like afatinib might be more effective than reversible inhibitors like lapatinib, there are concerns regarding the irreversible blockage of ErbB2 in healthy organs.[44] Further comparative pre-clinical and clinical studies in UBC are required.

In conclusion, *ERBB2* mutations occur in a subset of UBC and impact proliferation, signaling, gene expression and predict a greater response to lapatinib. If confirmed in the clinical setting, this may lead the way toward personalized treatment of advanced UBC.

Materials and Methods

Bladder cancer cell lines

The cell lines 5637, HT1197, HT1376, J82, RT4, SW780, T24, TCCSUP, and UM-UC3 were purchased from ATCC, and 647V, BC-3C, BFTC-905, CAL-29, JMSU-1, KU-19–19, RT112, SW1710, and VM-CUBI were purchased from DSMZ. 253J, TSU-PR1, UM-UC14 and WH were a gift from Dr Jer-Tsong Hsieh (UT Southwestern Medical Center, Dallas, TX), while 94-10, 96-1, 97-1, 97-7, 97-18, 97-24, DSH1, JO'N, SD, VM-CUBII, and VM-CUBIII were obtained from Dr Margaret

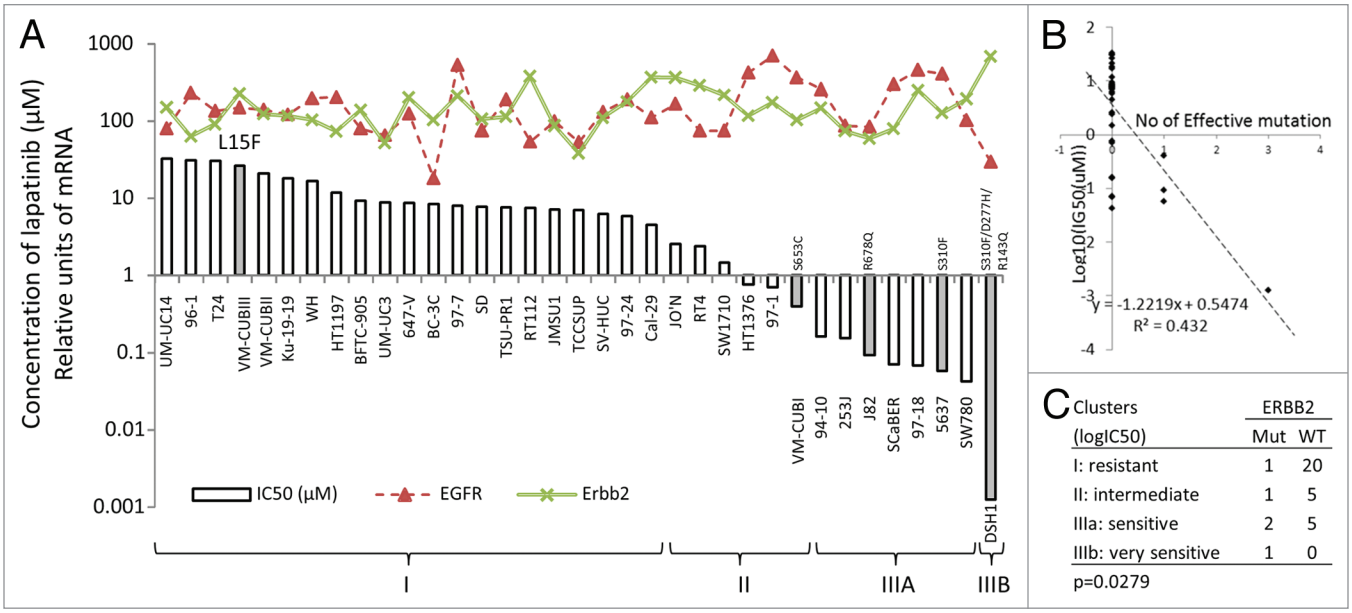

Figure 5. Response to lapatinib by *ERBB2* mutational status. (**A**) Cluster according to the sensitivity to lapatinib (upper panel) and IC_{50} for the cell lines as well as the relative expression levels of EGFR and ErbB2 (lower panel). The gray IC_{50} bars refer to cell lines with *ERBB2* mutation. DSH1 containing three *ERBB2* mutations was found to be the most sensitive cell line among all (group IIIB). (**B and C**) Correlation slope and contingency table of the numbers of *ERBB2* mutations and the sensitivity of cell lines to lapatinib, showing that the sensitivity was significantly increased in cell lines with *ERBB2* mutations.

A Knowles (Cancer Research UK Clinical Centre). All cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI-1640 medium) or Dulbecco's modified Eagle medium, which were supplemented with 10% fetal bovine serum and 1% non-essential amino acids. Cells were grown for one week in a humidified incubator at 37 °C and 5% CO_2 before the respective experiment, and planted at a density of 1000 cells/well in a 96-well plate.

Screening for ERBB2 and H-RAS mutations

Cells were lysed and the total RNA was extracted using RNeasy extraction kit (Qiagen) according to the manufacturer's instructions. RNA quality was assayed on an Agilent Technologies 2100 Bioanalyzer. For RNA sequencing, mRNA was purified from 1 μg of total RNA and retro-transcribed into a cDNA library. The pooled cDNA libraries were amplified on an Illumina cBot instrument, and then sequenced by Illumina HiSeq2000 sequencer using the pair end module. Reads were mapped to the human genome (hg18) using TopHat with default parameters. RefSeq transcript levels (FPKMs) were then quantified using CuffLinks, with upper-quartile normalization and sequence-specific bias correction.[45] Only missense and nonsense variants not listed in dbSNP 132 were retained for further analysis. All *ERBB2* and *H-RAS* mutations were confirmed by Sanger sequencing.

ERBB2 and BC proliferation

Wild-type human *ERBB2* was amplified using the primers 5′-GCTCGAGGCC GCAGTGAGCA CCATGGAGCT GGCGGCCTTG TG-3′ and 5′-GTCTAGATCA CACTGGCACG TCCAGACCC-3′ and the plasmid pDONR223-ERBB2 (Addgene plasmid 23888) as the template. Plasmids containing the mutated *ERBB2* were synthesized by introducing the desired mutations in one of the primers and performing the standard PCR with the wild-type *ERBB2* as template. The plasmid's DNA sequence was confirmed by Sanger sequencing.

In order to evaluate the role of the wild-type and mutated *ERBB2* in UBC proliferation, we performed knockdown experiments using shRNA plasmids (Open Biosystems) with three *ERBB2*-targeting shRNAs (sh1, sh4, sh6). The plasmid DNAs were inserted into the T-vector (Promega) and then transferred into the lentiviral vector pLvx-puro (Clontech Laboratories), as described above.

Activation status and cell growth pattern

Next, we wanted to test the phosphorylation/activation and the growth pattern in presence or absence of *ERBB2* mutations. For this purpose, a transient transfection was performed on 293FT cells (Life Technologies). This cell line was chosen for its high transfectability, fast growth, high production of viral titers and proteins. The cells were transfected with the plasmid containing the respective *ERBB2* sequences along with two helper/packaging plasmids which encode the structural and envelope proteins (ratio 4:2:1). The resulting viruses were collected at the time of their highest concentration (after 48–72 h) and used to infect RT112 cells. RT112 is an UBC cell line that is characterized by high expression of ErbB2. It has no known gain-of-function mutation involving the ErbB2 signaling pathway, which was found to be a good characteristic to investigate ErbB2 phosphorylation/activation.

Western blots were performed on extracts from 293FT and RT112 cells to assess the phosphorylation of ErbB2 and AKT(S473) in both cell lines. For this purpose, cells were washed

twice with cold phosphate-buffered saline and then collected using cell lifters. Cell pellets were lysed in 1× sodium dodecyl sulfate-PAGE (SDS-PAGE) loading buffer containing 50 mM sodium fluoride and 0.4 mM sodium orthovanadate. After boiling and centrifugation for 5 min, supernatants were used for SDS-PAGE.

The protein concentration was assayed using the Bradford method. Proteins were blotted to PVDF membranes. Antibodies to ErbB2 (Santa Cruz Biotechnology), AKT (Cell Signaling), β-Actin (Santa Cruz Biotechnology), β-Tubulin (Santa Cruz Biotechnology), phosphorylated residues (Cell Signaling Technology) and secondary antibodies linked with Horseradish peroxidases (Santa Cruz Biotechnology) were diluted in Tris-buffered saline with Tween-20 and 5% non-fat milk. For staining, cells were washed twice with PBS and fixed with 4% formaldehyde for 30 min. After a brief washing step with PBS, cells were stained with 0.05% crystal violet in 50% ethanol solution for 30 min. Subsequently, cells were rinsed in flowing water for 10 min and air-dried. The staining was extracted by 10% acetic acid, and its intensity was assayed by the light absorbance at 595 nm.

Gene expression analysis and hierarchical clustering

To evaluate effects of *ERBB2* mutations on downstream gene expression, RNA sequencing results from RT112 cells expressing the vector, wild-type, and mutant *ERBB2* (S310F and S653C) were analyzed by gene hierarchical clustering. The data of the four arrays were filtered by their readout, and a total of 10435 genes with 2 times change between any two groups were applied. After logarithmically transformed data were centered to the median and normalized, the clustering was performed with centroid, complete, and average linkages with uncentered Pearson correlation. Clusters and the array tree were prepared by Gene Cluster 3.0 and Java Treeview.[46,47] Gene clustering by functional annotation was executed at the website (http://david.abcc.ncifcrf.gov).[48,49] Statistical testing on a contingency table was performed using the Fisher exact test. Correlation of variables was assessed with the Pearson correlation coefficient.

For confirmatory qPCR, total RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase and random hexamer primer (Promega). Recombinant RNasin (Promega) was added to prevent RNA degradation. After the reaction, RNA was digested by RNase H (Clontech Laboratories). qPCR was performed on a 7500 Fast Real Time PCR System (Life Technologies) using the SYBR Green PCR mix (Life Technologies) and the standard two-step amplification. Specificity was verified by dissociation curves. The results were analyzed using relative quantification to the house keeping gene HSP90AB1.

Response to lapatinib

Microtetrazolium (MTT) assays were performed to assess the concentration of lapatinib (LC Laboratories), afatinib (Selleck Chemicals), and AZD6244 (inhibitor of MEK1 kinases, syn. Selumetinib, Selleck Chemicals) necessary to inhibit cell viability of 50%. Briefly, cells were treated for 48 h with different concentration of reagents ranging from 0.01 to 100 μM. Graphpad Prism software was used to calculate the IC_{50} value which was found to be 0.75 μM for lapatinib, 0.75 μM for afatinib and 4 μM for AZD6244.

The infected RT112 cells were cultured for 7 d and subsequently incubated with 4 μM AZD6244 or 0.75 μM lapatinib or 0.75 μM afatinib. As control, there were 5 wells with medium only and 5 wells with 0.1% DMSO for each plate. After 48 h of incubation, the presence of mini-foci was evaluated under the microscope. The relative numbers of viable cells were assessed before and after treatment using Celltiter Blue® Cell Viability Assay (Promega).

Disclosure of Potential Conflicts of Interest

The authors disclose no potential conflicts of interest and have no financial support to declare.

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